Early responses of human periodontal ligament fibroblasts to cyclic and static mechanical stretching

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Abstract: OBJECTIVE To compare the mechanotransduction caused by cyclic and static mechanical strains in human periodontal ligament fibroblasts (hPDLFs) cultured under identical conditions. MATERIALS AND METHODS hPDLFs, originating from the same donors, were exposed either to cyclic or to static tensile strain using specially designed devices and under identical culture conditions. Activation of all members of mitogen-activated protein kinases (MAPKs) was monitored by western immunoblot analysis. Expression levels of immediate/early genes c-fos and c-jun were assessed with quantitative real-time polymerase chain reaction. RESULTS Time course experiments revealed that both types of stresses activate the three members of MAPK, that is ERK, p38, and JNK, with cyclic stress exhibiting a slightly more extended activation. Further downstream, both stresses upregulate the immediate/early genes c-fos and c-jun, encoding components of the activator protein-1 (AP-1), a key transcription factor in osteoblastic differentiation; again cyclic strain provokes a more intense upregulation. Six hours after the application of both strains, MAPK activation and gene expression return to basal levels. Finally, cells exposed to cyclic stress for longer periods are distributed approximately perpendicular to the axis of the applied strain, whereas cells exposed to static loading remain in a random orientation in culture. CONCLUSION The findings of the present study indicate similar, although not identical, immediate/early responses of hPDLs to cyclic and static stretching, with cyclic strain provoking a more intense adaptive response of these cells to mechanical deformation.

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Summary

Periodontal ligament (PDL) is continuously exposed to different types of mechanical forces, such as cyclic or static deformation, caused by occlusion and mastication as well as during orthodontic treatment. These stresses activate immediately several signaling pathways and transcription factors, thus regulating remodeling and differentiation. The immediate reaction of PDL fibroblasts to cyclic or static stress was tested so far in different experimental setups. Aim of this study was to compare the mechanotransduction caused by these two types of mechanical forces in human PDL fibroblasts. Time-course experiments revealed that both types of stress activate through phosphorylation all three members of mitogen-activated kinases (MAPK), with cyclic stress exhibiting a slightly more extended activation over static stress. Both stress modes upregulate the immediate-early genes *C-FOS* and *C-JUN*, encoding components of the activator protein-1 (AP-1). However, cells undergoing cyclic stress presented a more intense expression of both genes. These data indicate similar immediate/early responses of PDL fibroblasts to cyclic and static stress on the level of all three members of mitogen-activated kinases (MAPK). Furthermore, cyclic stress seems to have a significant higher impact on the expression levels of both *C-FOS* and *C-JUN* genes when compared to static stretch.

Introduction

Periodontal ligament (PDL) is a highly specialized, connective tissue located between the tooth-root and the alveolar bone. The predominant cell type in this tissue are PDL fibroblasts (PDLF), which are capable for an osteoblastic differentiation, thus contributing to several processes including repair and regeneration, as well as remodeling of the surrounding hard tissue (Lekic and McCulloch 1996). PDLF osteoblastic differentiation can be achieved in response to a variety of extracellular stimuli, including mechanical strain (Arceo et al. 1991; Basdra et al. 1995; Carnes et al. 1997). Specifically, they are subjected to both cyclic forces caused by occlusion and mastication, as well as to static forces during orthodontic tooth movement (Mao 2010).

During orthodontic treatment continuous forces of different magnitude and duration are applied. Both fixed and removable appliances can be employed, the force pattern of whom differs significantly. Removable appliances exert intermitted forces to the teeth whereas fixed appliances produce continuous forces. Animal studies have shown that continuous, as well as intermittent forces produce the same orthodontic tooth movement (Oates et al. 1978; Reitan 1951; Steedle et al. 1983). All types of removable appliances such as functional, headgears etc. produce equivalent tooth movement with less than full time wear (Boecler et al. 1989; Graber 1985; Chateau et

al. 1983), since even a short exposure to mechanical loading can provoke bone remodeling (O'Connor et al. 1982). Fixed appliances exert continuous constant forces for a significantly extended amount of time, through the use of elastomeric chains, coil springs, and closing loops, before the force decays either due to force relaxation of the force applying mechanism or through tooth movement which decreases the magnitude of deformation of the applying medium.

The total mechanical loading exerted to the teeth by the aforementioned factors is converted into a cellular response (i.e. mechanotransduction) based on a complex network of sensing molecules (e.g. integrins). Stress fiber formation and focal adhesions organized by Rho family of Ras-related GTPases and activation of several signaling pathways, including the family of mitogen activated protein kinase (MAPK) cascades (i.e., the ERK, p38 and JNK), activate several specific transcription factors. One of the main transcription factors regarding osteoblastic differentiation is AP-1 (activator protein-1), which is involved in the regulation of osteoblast-specific genes, including alkaline phosphatase (ALP), collagen type I or osteopontin (OPN) (Franceschi et al. 1994). AP-1 is a homo-/heterodimeric complex consisted of members of the FOS and JUN families of transcription factors, controlling gene expression by binding to specific motifs in the regulatory regions of target genes (Whitmarsh and Davis 1996). AP-1 is important for the immediate cellular response to external stimuli (Karin and Minden 1997; Kyriakis 1999). In this vein, it has already been reported that static mechanical deformation of PDLF upregulates *C-JUN* and *C-FOS* (Kletsas et al. 2002), upregulates and activates RUNX2 via the ERK

pathway (Ziros et al. 2002) and increases AP-1 binding in the promoter of ALP (Peverali et al. 2001). Furthermore, cyclic mechanical stimulation in these cells activates ERK, JNK and p38 MAPK in a RhoK-dependent manner, upregulates *C-FOS*, and stimulates the expression of ALP, an early marker of osteoblastic differentiation (Jeon et al. 2009; Konstantonis et al. 2014).

Several studies have already focused on the immediate/early effects of cyclic or static forces on PDL fibroblasts. However, the majority of these studies have been performed using a variety of experimental protocols, regarding different force magnitude, duration and frequency, as well as different culture conditions (Carano and Siciliani 1996; Konoo et al. 2001; Konstantonis et al. 2014; Li et al. 2013; Mao 2010; Ren et al. 2015; Yamaguchi et al. 2002). Consequently, different, and in many cases contradictory, data have been reported. Accordingly, aim of this study was to compare the immediate/early effect of cyclic and static forces of the same magnitude on PDL fibroblasts cultured under identical conditions.

Materials and Methods

Cells and culture conditions

Human teeth from normal donors were extracted in the course of orthodontic treatment after having the approval of Bioethics' Committee of the National Centre for Scientific Research "Demokritos" (No 240/2013-1640). All donors signed an informed consent. PDL tissue explants were used to develop primary cultures of fibroblasts as previously described (Kletsas et al. 1998; 2002). In brief, the PDL

tissue attached to the apical third of the root surface was scraped off, cut into small pieces and placed in tissue culture dishes. The cells released from these tissues were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Biochrom AG, Berlin, Germany) and 10% (v/v) Fetal Bovine Serum FBS, from Gibco BRL (Invitrogen, Paisley, UK) in an environment of 5% CO_2, 85% humidity, and 37^{o}C and were subcultured when confluent by using a trypsin/citrate (0.25% / 0.30%, w/v) solution. PDL fibroblasts have been characterized by their spindle-like (fibroblastic) morphology and their ability of osteoblastic differentiation in response to an osteogenic medium (data not shown). Young (early-passage cells) were used at passages 3-6. Cells from five different donors were used in this study and each experiment has been reproduced in cells from at least two donors.

Application of mechanical stretching

Cyclic tensile strain was applied to hPDLF with a specially designed device, as described previously (Neidlinger-Wilke et al. 2001). Briefly, PDL fibroblasts were plated onto deformable and optically transparent silicone dishes, which were pre-coated with fibronectin from bovine plasma (Sigma, St. Louis, MO, USA) (25 ng/ml in in 0.5M NaCl- 50mM Tris-HCl, pH=7.5). Cells were maintained for 48h in DMEM supplemented with 10% FBS to adapt to culture conditions and 24h prior to mechanical stretching their medium was aspirated and replaced by fresh DMEM supplemented with 10% FBS. Cyclic strain (extension 8%, frequency 1Hz), i.e. under conditions falling within the range of physiological tissue deformation (Dong-Xu et al. 2011), was applied to the cells for the indicated time periods. Static tensile strain

(extension 8%) was applied with a novel in-house designed device and prepared by Controla (Advanced Technology Equipment, Athens, Greece) (Fig.1). In this device the same silicone dishes were used and cells were cultured under identical conditions.

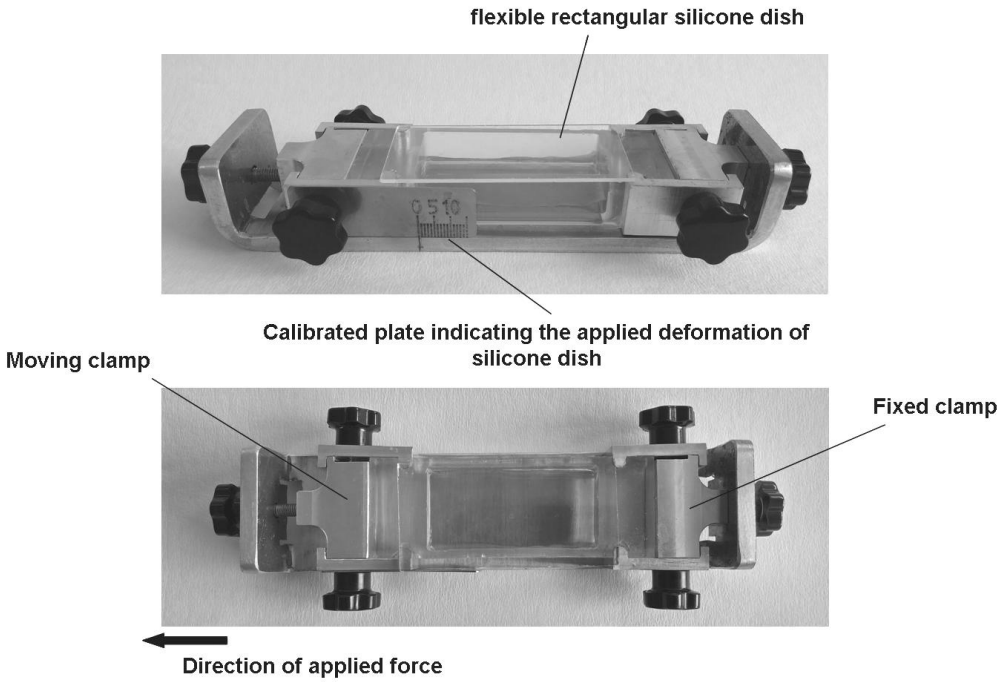


Figure 1. Static stretching apparatus. The rectangular flexible silicone culture dish is held at one end by a fixed clamp and the opposite end by a moving clamp. Strain magnitude can be modified manually by the knob of the moving clamp. A calibrated plate, attached to the moving clamp designating the deformation applied to the flexible silicone dish.

Western immunoblot analysis

The Western immunoblot analysis was performed as described previously (Konstantonis et al. 2013). Cells were washed with ice-cold tris buffered saline (TBS: 10mM Tris-HCl pH=7.4, 150mM NaCl) and scraped immediately in hot 2x SDS-PAGE sample buffer [125mM Tris-HCl pH=6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 125mM β-mercaptoethanol, 0.02% (w/v) bromophenol blue] supplemented with protease- and phosphatase-inhibitor cocktails (Sigma). Cell lysates were boiled for 3min, sonicated for 15s, clarified by centrifugation, aliquoted and stored at - 80^{o}C until use. The samples were separated on SDS-PAGE and the proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% (w/v) non-fat milk in 10mM Tris-HCl pH=7.4, 150mM NaCl, 0.05% Tween-20 (TBS-T) buffer and were incubated overnight at 4^{o}C with the appropriate primary antibodies. Antibodies against p38, phospho-p38 (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK were purchased from Cell Signaling Technology (Hertfordshire, UK). The antibodies against panERK and phospho-ERK1/2 (Thr202/Tyr204) were from BD Pharmingen (Bedford, MA); the PanActin antibody was from Neomarkers, Lab Vision Corporation (Fremont, CA, USA). After washing with 5% non-fat milk, the membranes were incubated with the respective secondary Horseradish peroxidase-conjugated antibody (Sigma) for 1.5h, washed again twice with 5% non-fat milk and once with TBS-T and finally the immunoreactive bands were visualized on Kodak-X-OMAT AR film by chemiluminescence (ECL kit), according to the manufacturer's (Amersham Biosciences) instructions. In all cases actin was used as a loading control.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR), as previously described (Konstantonis et al. 2013). Total RNA from unstimulated (control) and cyclically/statically-stretched hPDL fibroblasts was isolated using Trizol reagent (Life Technologies, Europe, BV). First strand complementary DNA (cDNA) was synthesized from 500ng of the total RNA using PrimeScript^{TM} RT Reagent Kit according to the manufacturer's instructions (Takara Bio Inc, Tokyo, Japan). 5μL of the cDNA (1:25) per sample was subjected to qRT-PCR by using the KAPA SYBR FAST qPCR kit (KAPA Biosystems, Massachusetts, USA). The reaction was carried out in an MX 3000 P QPCR Systems Cycler (Stratagene, La Jolla, USA) under the following conditions: denaturation program (95^{o}C for 3min), amplification and quantification program repeated 45 times (95^{o}C for 3s, 60-62^{o}C for 20s, 72^{o}C for 10s), melting curve program (60-95^{o}C with a heating rate of 0.1^{o}C/s and a continuous fluorescence measurement) and finally a cooling step to 55^{o}C. Data analysis was performed with MxPro QPCR software. Cycle threshold (C_t) values of each target gene were normalized to that of the house-keeping gene GAPDH. The ΔΔCt method was used to evaluate the relative mRNA expression of each gene. The primers used for amplification were *C-FOS*: forward 5'-AGA ATC CGA AGG GAA AGG AA-3', reverse 5'-CTT CTC CTT CAG GTT GG-3', *C-JUN*: forward 5'-CAC GTT AAC AGT GGG TGC CA-3' reverse 5'-CCC CGA CGG TCT CTC TTC A-3' and GAPDH: forward 5'-GAG TCC ACT GGG GTC TTC-3', reverse 5'-GCA TTG CTG ATG ATC TTG GG-3'.

Statistical Analysis

Hierarchical two-level linear mixed models were used to examine the effect of cell stretching (cyclic vs static) and time (15min to 6h) (predictor variables) on the expression of *C-FOS* and *C-JUN* separately, which accounted for the nested nature of the data (observations within donors). Model fit and data normality assumptions were checked through Kernel density residual plots and normal Q-Q plots. Natural logarithms of *C-FOS* and *C-JUN* expression were used in the regression model to allow for normality assumptions.

The level of significance was pre-specified at a = 0.05. All statistical analyses were conducted with STATA® version 14.1 software (Stata Corporation, College Station, Texas, USA).

Results

Response of young periodontal ligament fibroblasts to cyclic or static mechanical stimulation

Young (early-passage) human PDLF, plated on fibronectin-coated silicone dishes, were subjected to cyclic strain (extension 8%, frequency 1Hz) or to static deformation (extension 8%), for various time-points. In Fig. 2 can be seen that, in contrast to control cells, which were randomly distributed, the cells subjected to cyclic strain for 16h tented, to a significant extent, to be aligned perpendicularly to the direction of the force applied. On the other hand, the cells subjected to static stretching for the same period remained randomly distributed in the culture dish, similarly to control cultures.

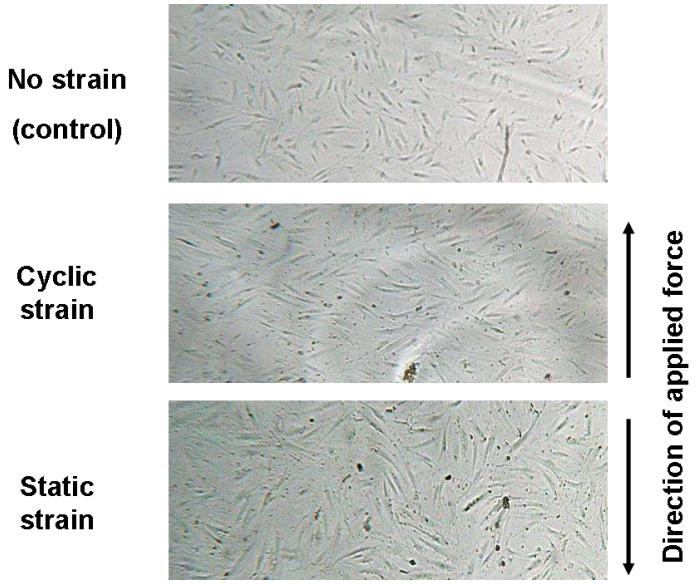


Figure 2. Static and cyclic tensile stretching has differential effect on the orientation response of hPDL fibroblasts. Young hPDL fibroblasts plated onto fibronectin-coated silicone dishes were subjected either to cyclic (extension 8%, frequency 1Hz) or to static tensile strain (extension 8%) for 16h and were photographed under an inverted microscope. Cyclically stretched cells are aligned nearly perpendicularly to the direction of the applied force (black arrows), whereas cells subjected to static strain, for the same period, remained randomly distributed in the silicone culture dish similarly to their unstimulated counterparts (control).

Activation of MAPK in response to cyclic and static tensile strain

Activation of MAPK pathways is a major immediate cell response to numerous external stimuli, including mechanical deformation (Konstantonis et al. 2014; Kyriakis 1999). Here, we exposed PDLF to cyclic or static tensile strain for various time points (up to 180min) and the activation of these pathways was evaluated by Western analysis. In all subsequent experiments we compared the responses of cells from the same donor to both types of stress; cells from at least three different donors were used in these experiments and all gave similar results. As can be seen in Fig. 3, and in accordance with previous findings (Konstantonis et al. 2014), cyclic stress activated all three pathways (ERK, p38 and JNK) 15min after stretching application. This activation (i.e. phosphorylation) remained for at least 60min and then returned to basal levels at 180min of stimulation. Interestingly, static tensile strain also activated these pathways 15min after cells' extension. However, this activation lasted at least 30min after stress application and was lost at 60min of stimulation.

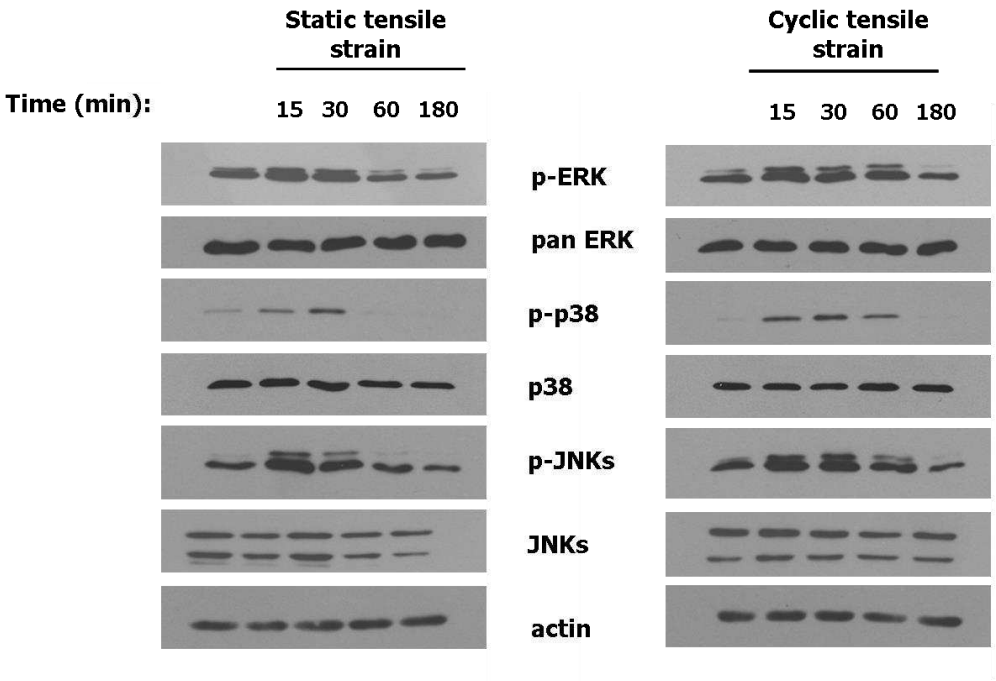


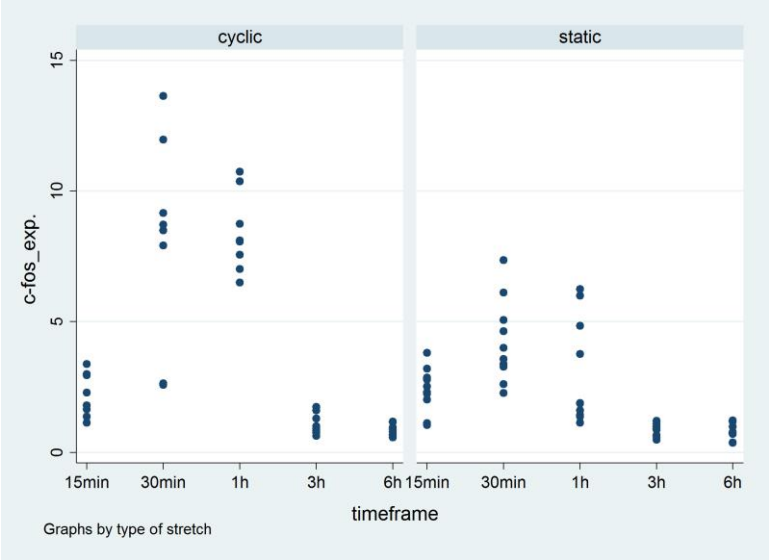
Figure 3. Human PDL fibroblasts respond readily to both static and cyclic tensile deformation. Cell lysates from both statically and cyclically stretched cells were collected at the indicated time points and the activation of ERK, p38 and JNK/SAPK MAPK kinases was assessed by Western blot analysis. Actin was used as a loading control. Representative blots from similar experiments performed in cultures from 3 donors are depicted here.

Regulation of *C-FOS* and *C-JUN* gene expression by cyclic and static tensile strain

Further downstream of MAPK activation we studied the effect of the two types of stretching on the expression of the immediate/early genes *C-FOS* and *C-JUN*, encoding members of the AP-1 transcriptional complex, the latter being important for the regulation of expression of osteoblast-specific genes. In terms of time frame comparison, both cyclic and static stretching activates *C-FOS* and *C-JUN* as soon as 15min after stress application. Gene expression after both types of stretching returned to the basal levels after 3-6h of stimulation (Fig. 4). In all experiments performed with cells from different donors the peak of activation was at approx. 30min for *C-FOS* and 60min for *C-JUN*. There was strong evidence that static stretching produced significantly lower expression of *C-FOS* in the multivariable model (β=-0.40, 95%CI: -0.59, -0.21; $p<0.001$) after adjusting for time. Cell stretching for 15 minutes (β=-0.87, 95%CI: -1.17, -0.57; $p<0.001$), 3 hours (β=-1.71, 95%CI: -2.02, -1.41; $p<0.001$) and 6 hours (β=-1.86, 95%CI: -2.16, -1.56; $p<0.001$) resulted in significantly lower *C-FOS* expression compared to 30 minutes evaluation, respectively. No interaction was detected ($p=0.93$) (Table 1).

For *C-JUN* expression again static stretching was associated with significantly lower gene expression (β=-0.27, 95%CI: -0.35, -0.20; p<0.001) when the effect of time was accounted. Cell stretching for 1 hour resulted in higher gene expression (β=0.40, 95%CI: 0.29, 0.51; p<0.001) when compared to 30 minutes stretching after adjusting for type of stretching. On the other hand 15 minutes stretching (β=-0.26, 95%CI: -0.37, -0.15; p<0.001), as well as 3 hours (β=-0.17, 95%CI: -0.28, -0.06; p<0.003) and 6 hours stretching (β=-0.32, 95%CI: -0.43, -0.21; p<0.001) revealed significantly lower *C-JUN* expression, respectively and after controlling for type of stretching. No interaction was present (p=0.24) (Table 2).

A.



B.

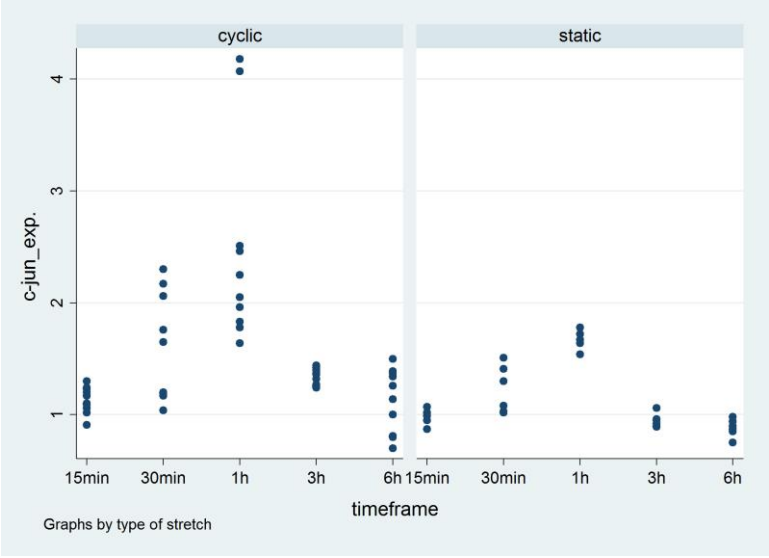


Figure 4. *C-FOS* (A) and *C-JUN* (B) expressions by type of stress and across time. Both types of strain activate both genes as soon as 15min after stress application. Gene expression reached its peak at 1h for *C-FOS* and 30min for *C-JUN*. Activation returned to basal levels after 3-6h.

Variable		**Two-level linear mixed model**		
		Coefficient (β)	**95% CI**	**p-value**
Type of stretch	Cyclic	Reference		
	Static	-0.40	-0.59, -0.21	**<0.001**
Time	30min	Reference		
	15min	-0.87	-1.17, -0.57	**<0.001**
	1h	-0.19	-0.49, 0.11	0.21
	3h	-1.71	-2.02, -1.41	**<0.001**
	6h	-1.86	-2.16, -1.56	**<0.001**
Stretch*Time				0.93

Table 1. Hierarchical two-level mixed linear regression with observed coefficients and 95% CIs for the effect of stretch and time on *C-FOS* expression (*C-FOS* in logarithmic scale).

Variable		Two-level linear mixed model		
		Coefficient (β)	**95% CI**	**p-value**
Type of stretch	Cyclic	Reference		
	Static	-0.27	-0.35, -0.20	**<0.001**
Time	30min	Reference		
	15min	-0.26	-0.37, -0.15	**<0.001**
	1h	0.40	0.29, 0.51	**<0.001**
	3h	-0.17	-0.28, -0.06	**0.003**
	6h	-0.32	-0.43, -0.21	**<0.001**
Stretch*Time				0.24

Table 2. Hierarchical two-level mixed linear regression with observed coefficients and 95% CIs for the effect of stretch and time on C-JUN expression (C-JUN in logarithmic scale).

Discussion

Periodontal ligament is continuously exposed to different types of mechanical forces, such as cyclic or static deformation, caused by occlusion and mastication as well as during orthodontic treatment (Mao 2010). Periodontal ligament cells and more

specifically periodontal ligament fibroblasts respond to these mechanical loads, transforming them to biochemical signals, e.g. activation of intracellular signaling pathways and expression of specific genes, eventually leading to PDL repair, remodeling, differentiation and regeneration. There is an increasing number of studies dealing with the response of PDL fibroblasts to cyclic and static mechanical stimulation. Among the most important targets are the activation of the MAPK signaling pathways and the regulation of the *C-FOS* and *C-JUN* genes, encoding members of the AP-1 transcription factor, the latter playing significant role in the regulation of osteoblast specific genes, such as *ALP*, collagen I or osteopontin (Kletsas et al. 2002). Different types of mechanical stimulation, of different duration, frequency and magnitude, have been used in these studies (Mao 2010). Furthermore, the same type of mechanical deformation has been studied by using different experimental setups or devices. For example, the application of mechanical forces has been studied by cell centrifugation, in cells cultured on flexible-bottom surfaces extended by vacuum or using uniaxial strain (Yamaguchi et al. 2002),(Chiba and Mitani 2004). Similarly, static stretching has also been studied under varying conditions of culture and mechanical deformation. Finally, there are no data in the literature comparing the immediate/early responses of these cells to the application of cyclic or static mechanical loading. To this end, we cultured the cells under identical conditions, i.e. on fibronectin-coated extendable silicone dishes, and tensile stretching has been applied in a well-established device for cyclic deformation (Neidlinger-Wilke) and a new home-made device for static extension (Fig. 1). First, we studied the activation of the MAPK signaling pathways as they represent a major initial

cellular response to a variety of exogenous stresses (Karin and Minden 1997). Previous studies have shown the activation of MAPK in PDL fibroblasts in response to a cyclic or static mechanical deformation of varying conditions (type of stress, magnitude, duration or frequency) (Kletsas et al. 2002; Konstantonis et al. 2014; Kook et al. 2011; Peverali et al. 2001; Ziros et al. 2002). Here, by using PDL fibroblasts cultured under identical conditions, we showed that both cyclic and static stretching of the same magnitude activate all three MAPK pathways (ERK, p38 and JNK). Interestingly, all pathways are rapidly activated with both types of stress and the only difference found was that the phosphorylation of these kinases was slightly more extended after cyclic stress, as compared to static loading.

The transcription factor AP-1 plays a central role in the regulation of genes that are activated early in osteoblastic differentiation (Peverali et al. 2001),(Stein et al. 1996);(Franceschi 1999). In addition, *C-FOS* upregulation is considered as a classical response to mechanical stress. Previous studies have shown in PDL fibroblasts the upregulation of the *C-FOS* and *C-JUN*, members of the AP-1 complex, in response to mechanical stimulation, as well as the potentiation of AP-1 binding in the promoter of the ALP gene, a marker of osteoblastic differentiation (Kletsas et al. 2002; Konstantonis et al. 2014; Kook et al. 2011; Peverali et al. 2001). Notably, it has been shown that members of the MAPK signaling pathways control the expression of AP-1 components after mechanical stimulation (Kletsas et al. 2002; Kook et al. 2011; Peverali et al. 2001). The comparative study performed indicates that both static and cyclic stretching upregulate the *C-FOS* and the *C-JUN* genes. The peak of activation

was observed 30-60 minutes after stimulation, while the expression returned to the basal levels after 3-6 hours. However, cyclic stretching provokes a significantly higher induction of both genes in agreement with the more extended MAPK activation. The observation of the similar kinetics in gene expression, although cyclic stretching continued for longer time periods, clearly indicates an adaptive response. In this vein, we have shown that a long-term cyclic tensile stretching provokes an alignment of PDL fibroblasts in a direction, approx. perpendicular to the stretch direction, in agreement with the results of Neidlinger-Wilke et al. (2001), on skin fibroblasts and osteoblasts (Buck 1980). In contrast, after the exposure of PDL fibroblasts to static tensile stretching, of the same duration and magnitude, the cells remain in a random orientation in culture, similarly to control (unstretched) cultures.

The findings of this study indicate that there are statistically significant differences between static stretch and cyclic stretch. Static stress was found to provoke lower gene expression and protein activation compared to cyclic stretch. Given the fact that static stress simulates orthodontic treatment while cyclic stress mimics occlusal forces, future studies studying the impact of orthodontic forces on cell proliferation should consider the use of devices evoking static stretch. Thus, the results of previous studies performing cyclic stress as a simulation of tooth movement during orthodontic treatment might be questioned.

In summary the results of the present study, indicate that under identical culture conditions PDL fibroblasts, originating from the same donors, have similar

immediate/early responses when exposed to cyclic or static tensile stretching of the same magnitude, with cyclic strain provoking significantly more intense gene expressions. These findings suggest a major adaptive response and are in line with the altered orientation of cells after a long-term mechanical stimulation. However, the biochemical basis of this adaptive response to various type of mechanical deformation needs further investigation.

Competing interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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